Virology / Virologie

Polymerase chain reaction detection of greening bacterium (*Candidatus* Liberibacter asiaticus) and *Citrus mosaic virus* in citrus tissues, by means of a simplified template-preparation protocol

V.K. Baranwal, K.N. Gupta, and R.P. Singh

Abstract: Application of a simplified protocol for nucleic acid preparation for polymerase chain reaction (PCR) detection of greening bacterium (*Candidatus* Liberibacter asiaticus; Cla) and *Citrus mosaic virus* (CMBV) associated with citrus is described. Crude extracts of citrus tissues in NaOH – ethylenediaminetetraacetic acid solution, prepared without the use of liquid nitrogen, were spotted on a nitrocellulose membrane (NCM) and then eluted in water at 80 °C for 10 min. Both the greening bacterium and CMBV were detected by PCR from the eluted liquid from the spotted NCMs. The detection efficacy for the templates obtained in the NCM-eluted liquid was comparable with that of templates obtained with a multistep laboratory method or a commercial kit for nucleic acid preparation. The protocol is simple, inexpensive, rapid, and applicable to large-scale surveys of citrus trees. The spotted-membrane methodology can also be used for short-term sample storage for future testing or for long-distance sample transport to a detection laboratory.

Key words: Candidatus Liberibacter asiaticus, citrus, *Citrus mosaic virus*, detection method, polymerase chain reaction.

Résumé : Un protocole simplifié de préparation d'acide nucléique pour la détection, par réaction en chaîne de la polymérase, de la bactérie causant la maladie du dragon jaune (*Candidatus* Liberibacter asiaticus; Cla) et du virus de la mosaïque des agrumes (CMBV) présents chez les agrumes est décrit. Des extraits bruts de tissus d'agrumes, préparés dans une solution de NaOH et d'acide éthylènediamine-tétracétique, sans utiliser d'azote liquide, ont été appliqués sur une membrane de nitrocellulose pour être ensuite élués dans l'eau par un traitement thermique à 80 °C durant 10 min. Tant la bactérie responsable de la maladie du dragon jaune que le CMBV ont été détectés par réaction en chaîne de la polymérase lorsque le liquide élué de membranes a été utilisé. Les gabarits dans le liquide élué ont permis une efficacité de détection équivalente à celle obtenue avec une méthode de laboratoire comportant plusieurs étapes ou avec une trousse commerciale de préparation d'acide nucléique. Le protocole est simple, peu coûteux, rapide et utilisable pour l'examen à grande échelle des agrumes. La méthodologie d'application sur membrane peut aussi servir à l'entreposage à court terme des échantillons pour un examen subséquent ou pour leur transport sur longue distance jusqu'à un laboratoire de détection.

Mots-clés : Candidatus Liberibacter asiaticus, agrumes, virus de la mosaïque des agrumes, méthode de détection, réaction en chaîne de la polymérase.

Introduction

Citrus is an important fruit crop grown in more than 140 countries. Productivity is often affected by a number of graft-transmissible viruses and viruslike pathogens, namely eight groups of viruses, five groups of viroids, four types of prokaryotes, and a number of uncharacterized viruslike and decline-inducing agents (Garnsey 2002). Their detection in propagating material is an important requisite to ensure the production of healthy planting material. In India, the major viruses infecting citrus are *Citrus tristeza virus* (CTV), *Indian citrus ringspot virus* (ICRSV), and *Citrus mosaic virus* (CMBV) previously described as citrus yellow mosaic virus (Ahlawat and Pant 2004). CTV and ICRSV are RNA

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viruses belonging to the genera *Closterovirus* and *Mandarivirus*, respectively, and are detected easily by serological assays such as enzyme-linked immunosorbent assay (ELISA) (Cambra et al. 1991; Rustici et al. 2000). In contrast, CMBV is a bacilliform, nonenveloped DNA virus with viral particles of 130 nm × 30 nm (Ahlawat et al. 1996a) and containing a circular double-stranded genome of 7559 base pairs (bp); it is grouped in the genus *Badnavirus*, family *Caulimoviridae* (Huang and Hartung 2001). Polymerase chain reaction (PCR) is a preferred method of detection of CMBV (Baranwal et al. 2003) largely because badnaviruses are weak immunogens and because their detection by serological methods is not reliable. CMBV is widely distributed in India, especially in sweet orange (*Citrus sinensis* (L.) Osbeck) and pummelo (*Citrus grandis* (L.) Osbeck) (Ahlawat et al. 1996a; Ahlawat 1997). CMBV has also been observed in Rangpur lime (*Citrus limonia* Osbeck) rootstock (Baranwal et al. 2005). CMBV is transmissible to 13 citrus cultivars via grafting (Ahlawat et al. 1996a) and via insects such as the citrus mealybug (*Planococcus citri* (Risso) (Reddy 1997). Affected citrus trees produce significantly less fruit, juice, and ascorbic acid and have a shortened productive life (Ahlawat et al. 1996b).

Another graft-transmissible viruslike pathogen in citrus is the difficult greening bacterium (*Candidatus* Liberibacter asiaticus (Jagoueix et al.) Euzéby; Cla) (Ahlawat and Pant 2004) that causes greening or yellowing diseases in different citrus cultivars. It has been found in several African and Asian countries (including parts of the Arabian Peninsula) (da Graça 1991; Garnier and Bové 1993) and, recently, in the United States (Li et al. 2006). Cla can cause crop losses of 30%–100%, and citrus production may be eliminated in some production areas (da Graça 1991). The bacterium cannot be cultured; however, conventional and real-time PCR assays have been developed for its detection in infected plants and in the vector Asian citrus psyllid (*Diaphorina citri* Kuwayama) (Jagoueix et al. 1996; Baranwal et al. 2004; Hung et al. 2004; Li et al. 2006).

In most PCR assays developed for CMBV and Cla, the protocols for DNA-template preparation involve multistep extraction and precipitation with or without the use of phenol–chloroform (Baranwal et al. 2003; Li et al. 2006). Most often, expensive commercial kits are used for nucleic acid isolation for the PCR detection of these pathogens (Jagoueix et al. 1996; Huang and Hartung 2001; Baranwal et al. 2004). These protocols also require, for the grinding of tissues, liquid nitrogen that may not be readily available in most regional laboratories in developing countries. In recent years, novel approaches have been developed to avoid the use of organic solvents and to simplify the preparation of nucleic acid templates of plant viruses and viroids. These approaches include immunocapture with antibodies, immobilization on polypropylene or polystyrene surfaces (Rowhani et al. 1995; James 1999), use of various proteins for coating surfaces (Olmos et al. 1996), immobilization of plant sap on filter paper (Weidemann and Buchta 1998), premoistened N^+ nylon membrane (La Notte et al. 1997), and untreated nitrocellulose membrane (NCM) (Singh et al. 2004, 2006). Of these approaches, the simplified protocols with membrane-based nucleic acid extraction (Singh et al. 2004, 2006) have worked well for detection of viruses and viroids in herbaceous crops but have not been tested for woody plants such as citrus. Because the procedures described by Singh et al. (2004, 2006) do not require liquid nitrogen for the grinding of plant tissues and because there is no involvement of organic solvents, nor multistep extraction and precipitation, the methodology seemed best suited for our laboratory. Thus, our objective was to evaluate the feasibility of membrane-based nucleic acid extraction for preparation of DNA templates from citrus tissues for two groups of pathogens (viruses and bacteria). This paper reports the successful evaluation of a simple, inexpensive, and rapid method of template preparation for the PCR-based detection of two DNA-containing pathogens (CMBV and Cla) in citrus tissues.

Materials and methods

CMBV was maintained in *C. sinensis* and *C. grandis* plants, and Cla in *C. sinensis*, the respective natural hosts. Healthy citrus seedlings were grown from seed and grafted 1 year later with budwood infected with CMBV and Cla. All plants were maintained in a greenhouse at temperatures ranging from 35 to 28 °C (day and night, respectively) with a photoperiod of 14 h. Entire leaf blades were used for detection of CMBV, whereas only petioles and midribs were used for detection of Cla, because the latter pathogen is limited to the phloem. A membrane-based extraction method of nucleic acids of CMBV and Cla in sodium hydroxide – ethylenediaminetetraacetic acid (NaOH–EDTA) was employed for the preparation of nucleic acid templates (Singh et al. 2006), which were compared with those obtained with a commercial kit for detection of CMBV and Cla. In addition, these two methods, and a multistep method with an extraction buffer containing sodium sulphite (Singh et al. 2002; Baranwal et al. 2003), were compared for the detection of CMBV and Cla. All tests were repeated two times unless mentioned otherwise.

Direct extracts and membrane-eluted nucleic acid preparations

One hundred milligrams of leaf tissue from CMBV-infected plants or 100 mg of petiole and midrib of leaves tissue from Cla-infected plants were homogenized in 1 mL of an alkaline solution (NaOH, 50 $mmol \cdot L^{-1}$; EDTA, 2.5 $mmol \cdot L^{-1}$) (Turturo et al. 1998; Singh et al. 2006), using a sterilized mortar and pestle. No liquid nitrogen was employed for the grinding of tissues. The resulting extracts were incubated at room temperature (24–32 °C) for 15 min or centrifuged at 12 000 *g* for 10 min, and PCR was performed directly on the supernatants from both the CMBV and Cla treatments (termed direct extracts). Alternatively, 5 μL of sap were spotted on untreated NCMs (BAS 85; pore size, 0.45 μm; Schleicher & Schuell, Keene, N.H.) that were then dried for 30 min at 24–32 °C. Individual spots (4.0 mm) for each sample were cut out with a paper hole punch (Kangaro Industries, Ludhiana, India) and eluted in 30 μL of sterile distilled water by incubation at 80 °C for 10 min on a heat block. The liquid was collected by centrifugation (termed NCM-eluted extract). Volumes of 2.5, 5, 10, and 20 μL were used for detection of Cla and CMBV DNAs by PCR.

Evaluation of shelf life of spotted NCM discs for pathogen detection by PCR after short-term storage

Two sets of NCMs were spotted with leaf sap from either CMBV- or Cla-infected plants. The membranes were used after drying (day 0) or stored at room temperature (24–32 °C) for 1, 3, 7, 15, 30, or 60 days. Nucleic acids were eluted from five individual spots from each set of membranes (total 10 spots) per time point for each of the pathogens and served as templates for PCR amplification. After amplification, the PCR products were separated on agarose gels, stained with ethidium bromide, visualized under UV light, and photographed. Presence or the absence of specific bands on the gel was recorded.

Comparison of nucleic acid preparations with the membrane-based method, the multistep method, and a commercial kit

Total nucleic acids from *C. sinensis* infected either with CMBV or Cla were extracted by the multistep method with sodium sulphite (Baranwal et al. 2003) and with a commercial DNA-isolation kit (Plant DNeasy mini kit; Qiagen, Gmbh, Hilden, Germany), as per the manufacturer's protocol. DNA isolated with these two methods was compared with NCM-eluted material. In all (except NCM-eluted) cases, liquid nitrogen was employed for grinding to a fine powder 100 mg of tissue from CMBV- or Cla-infected plants as well as from healthy plants. The final DNA pellets of CMBV and Cla were dissolved in 100 μL of distilled water for the two procedures, and 5 μL of DNA samples were used as templates for PCR detection of CMBV and Cla. The PCR products were compared with those obtained with PCR detection of these two pathogens from 20 μL of NCM-eluted DNA templates.

PCR for detection of CMBV and Cla

Volumes used for PCR detection were as follow: (1) 5 μL of sap obtained after grinding leaf tissue infected with CMBV, or petiole and midrib tissue infected with Cla, in an alkaline NaOH–EDTA solution, for the direct extract method; (2) 2.5, 5, 10, or 20 μL for the NCM-eluted DNA; or (3) 5 μL of DNA isolated by means of a commercial kit or the multistep method with sodium sulphite were used for PCR in a 50 μL reaction mix containing: forward and reverse primers of CMBV (5′-GAGCTATTAGAAGGAATCTC and 5′-AACCAAGCTCTGATACCA, respectively) or of Cla (5′-TGGGTGGTTTACCATTCAGTG and 5′-CGCGACTTC-GCAACCCATTG, respectively), 0.1 μg each; *Taq* DNA polymerase (Promega, Madison, Wisc.), 5 U (1 U ≈ 16.67 nkat); 10× PCR buffer, 5 μL; dNTPs (Qiagen), 200 $\mu mol \cdot L^{-1}$ of each; and $MgCl_2$, 1.5 $mmol \cdot L^{-1}$. Samples were amplified for 30 cycles, using a Mastercycler® (Eppendorf, Germany). Each cycle consisted of denaturation at 94 °C (30 s), primer annealing at 54 °C for CMBV and 58 °C for Cla (60 s), extension at 72 °C (60 s), and a final extension of 10 min at 72 °C.

Ten microlitres of amplified product were separated by electrophoresis in a 1.5% agarose gel containing ethidium bromide at a concentration of 0.5 $\mu g \cdot mL^{-1}$ and were photographed under UV illumination with an imaging system (XR documentation system, Bio-Rad, Reading, Penn.).

Cloning and sequencing

The amplified DNA products of CMBV and Cla were excised and eluted from the gel with Qiaquick gel extraction kit (Qiagen). The purified PCR products were ligated into pGEM-T Easy vector (Promega). Competent *Escherichia coli* (Migula) Castellani & Chalmers (strain DH 5α) were transformed according to standard molecular biology procedures (Sambrook and Russel 2001). Recombinant clones were identified by restriction endonuclease digestion, and two clones of each pathogen were sequenced at Microsynth, Balgach, Switzerland. The Basic local alignment search tool (BLAST; National Center for Biotechnology Information, Bethesda, Md.) was used to compare the sequences with sequences in GenBank.

Results

Determination of optimal volume of NCM-eluted extract for PCR detection of DNA pathogens

Because herbaceous plant tissues and an RNA-pathogen template were used in the initial procedure reported by Singh et al. (2006), it was necessary, in the present study, to determine the effect of the volume of NCM-eluted extract on PCR detection for the CMBV and Cla DNA pathogens from citrus leaves (Figs. 1A and 1B, respectively). The results indicated that DNAs of these pathogens were absorbed and retained on the NCM and eluted with fewer inhibitors. However, the amount of NCM-eluted extract needed to yield prominent PCR-amplified bands varied with the two pathogens (viral and bacterial). For CMBV, the amount of NCM-eluted extract needed for PCR was in the range of 2.5–20 μL (Fig. 1A, lanes 2–13), with strong bands observed with volumes of 5–20 μL (lanes 4–10). For Cla, the amount of NCM-eluted extract needed for PCR was in the range of 5–20 μL (Fig. 1B, lanes 2–10), with the most intense band observed with 20 μL (lanes 2–4).

To further verify the suitability of the 20 μL volume for PCR detection, three citrus species (sweet orange, pummelo, and Rangpur lime) naturally infected with CMBV were tested. Twenty microlitres of NCM-eluted extracts of three separate samples from each species were used and, as shown in Fig. 2, CMBV-specific bands were observed in each case, and there was no amplification from healthy samples (lanes 4, 8, and 12). The bands were prominent in all the three species; with Rangpur lime, there was an indication of differential band intensity, suggesting a higher virus concentration in the third sample (lane 3) or, possibly, some inhibitors eluted in the first and second samples (lanes 1–2). The direct extracts of Cla or CMBV failed to yield any products when used as templates in PCR (Fig. 3, lanes 4–6), suggesting the presence of inhibitors of the *Taq* polymerase.

Comparison of a commercial kit, the membrane-based method, and the multistep method for template preparation

In a series of experiments, widely used methods of nucleic acid preparation were compared with the NCM-eluted extraction method for each pathogen type. The nucleic acid template from NCM-eluted samples gave strong amplification of CMBV DNA (Fig. 4A, lanes 1–3), whereas those prepared with a commercial kit (lanes 5–7) or the multistep

Fig. 1. Effect of varying volumes (20, 10, 5, and 2.5 µL) of nitrocellulose membrane (NCM)-eluted extract for detection of (A) *Citrus mosaic virus* (CMBV) and (B) greening bacterium (*Candidatus* Liberibacter asiaticus) by polymerase chain reaction. M, 1-kilobase DNA ladder; lane 1, healthy plant tissues; lanes 2–4, 20 µL; lanes 5–7, 10 µL; lanes 8–10, 5 µL; lanes 11–13, 2.5 µL; lane 14, positive control constituted from a colony of transformed *Escherichia coli*.

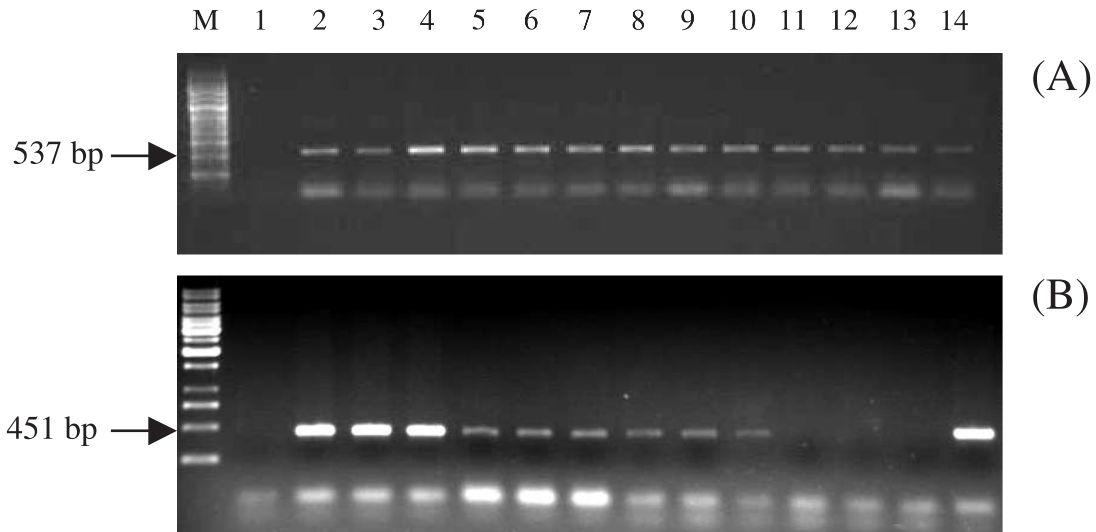

Fig. 2. Detection of *Citrus mosaic virus* (CMBV) from three naturally infected citrus species by polymerase chain reaction with 20 µL of nitrocellulose membrane (NCM)-eluted extract. Lane M, marker; lanes 1–3, Rangpur lime; lanes 4, 8, and 12, healthy plant tissues; lanes 5–7, pummelo; lanes 9–11, sweet orange.

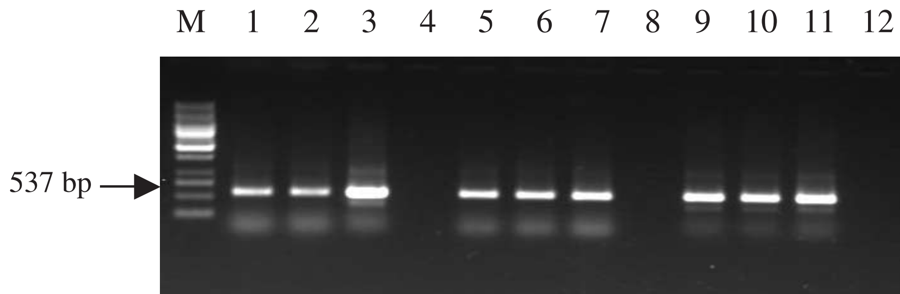

Fig. 3. Comparative detection of (A) *Citrus mosaic virus* (CMBV) and (B) greening bacterium (*Candidatus* Liberibacter asiaticus) by polymerase chain reaction on direct extract of sap in alkaline NaOH – ethylenediaminetetraacetic acid (EDTA) solution (lanes 4–6) and on the following nitrocellulose membrane (NCM)-eluted extract (lanes 1–3). Lane M, 1-kilobase DNA ladder; lane 7, extract from healthy plant tissues; lane 8, positive control constituted from a colony of transformed *Escherichia coli*.

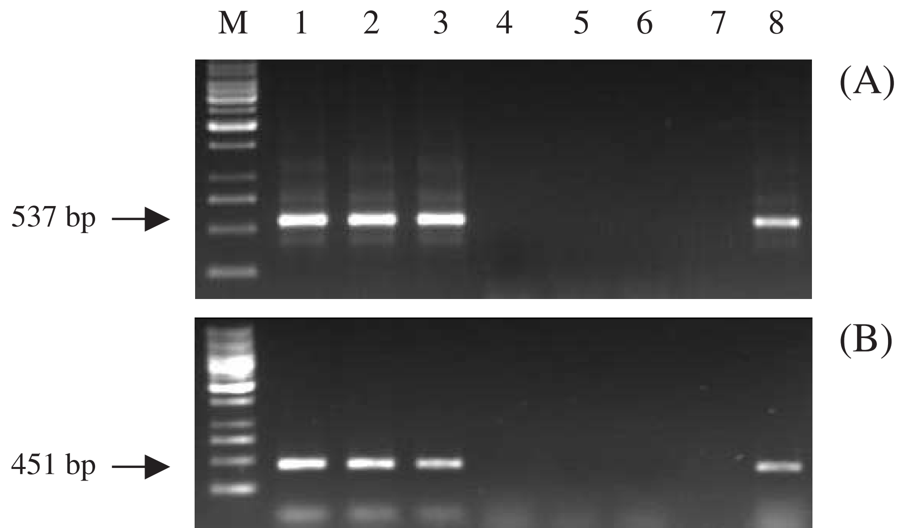

Fig. 4. Comparison of the detection of DNAs of (A) *Citrus mosaic virus* (CMBV) and (B) greening bacterium (*Candidatus* Liberibacter asiaticus) in sweet orange by polymerase chain reaction, following three DNA-templates preparation methods: (1) spotting of plant-tissue extracts in NaOH – ethylenediaminetetraacetic acid (EDTA) on nitrocellulose membrane (NCM), followed by elution in water under thermal treatment (lanes 1–3); (2) use of a commercial kit for DNA extraction (lanes 5–7); and (3) a multistep DNA-extraction method with sodium sulphite (lanes 9–11). Lanes 4, 8, and 12, extract from healthy plant tissues; lane 13, positive control constituted from a colony of transformed *Escherichia coli*; lane M, 1-kilobase DNA ladder.

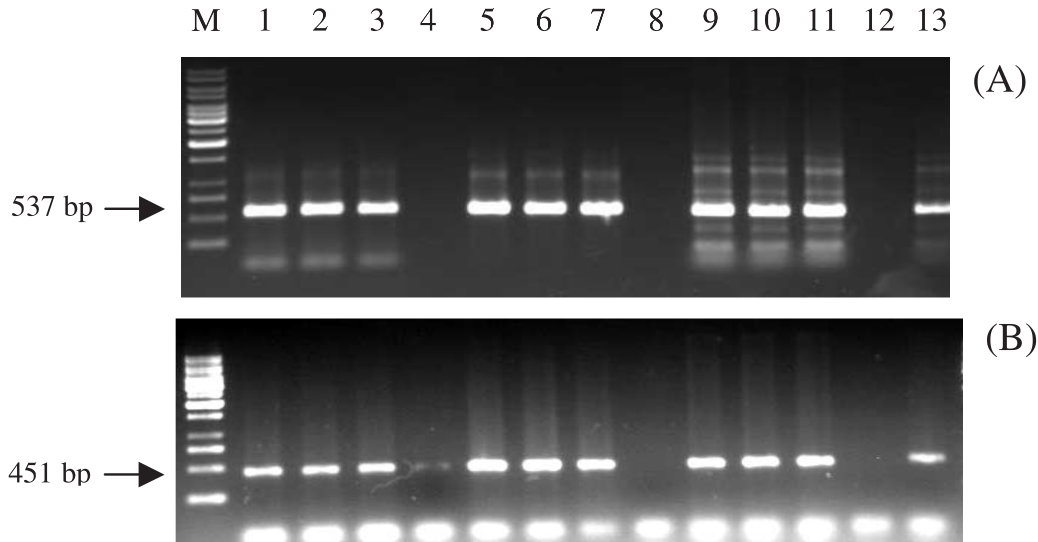

Table 1. Shelf life of nitrocellulose membrane (NCM) spotted with nucleic acids extracts from citrus tissues in NaOH – ethylenediaminetetraacetic acid (EDTA) solution until testing for detection of the DNA pathogens *Citrus mosaic virus* (CMBV) and greening bacterium (*Candidatus* Liberibacter asiaticus; Cla) by polymerase chain reaction (PCR).

		CMBV		Cla	
Sample No.	Shelf life of spotted NCM until testing (d)	PCR band intensity[a]	No. of positive spots[b]	PCR band intensity[a]	No. of positive spots[b]
1	0 (day 1)	+++	9	+++	9
2	3	+++	10	+++	8
3	7	++	8	+++	9
4	15	+++	9	++	9
5	30	+++	9	+++	8
6	45	+++	8	+++	9
7	60	++	9	++	8

[a]Band intensity of the PCR products was scored as follows: ++, moderate intensity; +++, good intensity.
[b]Ten spots were placed on each membrane.

method with sodium sulphite (lanes 9–11) were slightly more intense than the NCM-eluted samples. Similarly, results obtained with Cla bacterial DNA were most intense with the commercial kit and the multistep method with sodium sulphite, but the NCM elution delivered intense bands as well (Fig. 4B, lanes 1–3, 5–7, and 9–11 for NCM elution, commercial kit, and multistep method with sodium sulphite, respectively).

Evaluation of shelf life of spotted NCM for pathogen detection by PCR after short-term storage

The spotted samples of the extract prepared in NaOH–EDTA containing Cla and CMBV were tested by PCR immediately after preparation and then again at different days of storage. Both Cla and CMBV were detected by PCR from the eluted samples, and there was no obvious reduction in the band intensity. A total of 70 spots were tested each for Cla and CMBV pathogens. Among the 10 spotted samples, 1 or, rarely, 2 failed to be amplified after different time intervals (Table 1). In contrast, the DNA extracted with a commercial kit or the multistep method with sodium sulphite failed to amplify for CMBV and Cla after 3 days of storage at room temperature (results not shown).

Application of the final protocol for field evaluation of two pathogens

The final protocol of simplified template preparation was tested for symptom expression in 10 samples of Sathgudi sweet orange (naturally infected with CMBV), collected from two orchards in Andhra Pradesh (southern India), or Kinnow mandarin (*Citrus reticulata* Blanco) (naturally infected with Cla), collected from two orchards in Delhi (northern India).

CMBV was detected in all 10 samples, whereas Cla was detected in 8 of 10 samples. The two samples that tested negative for Cla were free from infection by greening bacterium; however, they were visually misdiagnosed because yellowing symptoms were present but apparently caused by nutritional deficiencies. The symptoms disappeared in these two plants upon correction of the nutritional deficiencies, whereas the other eight plants continued to show symptoms of Cla infection. The two healthy citrus plants were used as a negative control and did not show visual symptoms of the presence of the two pathogens, whereas positive control plants for these pathogens did.

Sequence determination of the amplified products

The cloned and sequenced product of CMBV was 537 bp in size and had an 81% sequence identity with the nucleotides of the corresponding region of 567 bp in CMBV GenBank accession No. AF347695. There were deletions at several places, which led to the reduction of 30 nucleotides in amplicon size. The cloned sequence of Cla was rather well conserved and had 96%–100% sequence identity with the nucleotides of Cla accession Nos. AY919311 (96%), AB008366 (96.4%), L22532 (98%), and DQ157274 (100%). The primer pairs used for detection of CMBV and Cla had 100% sequence identity with the sequences of these two pathogens in GenBank.

Discussion

Detection of CMBV and Cla can be reliably performed by PCR. One of the major steps in PCR-based detection of these and other pathogens is the preparation of nucleic acid templates. Direct extract (crude extract without NCM spotting and elution) of denatured sap (alkalinized through NaOH–EDTA extraction and heat treated) as well as eluted extract from spotted NCMs have been used successfully for viroid detection through RT-PCR in herbaceous plants (Singh et al. 2006), but the direct extract of denatured sap containing nucleic acids of Cla or CMBV was not suitable, in the present study, for detection of the DNAs by PCR. This failure may be due to the fact that the crude extract contains inhibitors that might interfere with PCR amplification of Cla and CMBV. The material eluted from the membranes was rather colourless because most of the pigments stayed on the membrane. It is likely that many inhibitors of PCR present in the sap extract also remained on the membrane, which made the eluted solution suitable for amplification of DNA from both the pathogens. The NCM-eluted nucleic acids from individual spots of CMBV and Cla gave amplifications that were comparable with those of nucleic acids extracted with a commercial kit. The amount of NCM-eluted extract needed for consistent amplification was different with CMBV and Cla. Only 5–20 µL of eluted denatured homogenate were required for CMBV detection, whereas 10–20 µL were required for the detection of Cla. CMBV infects all the tissues of citrus leaves compared with Cla, which is phloem limited, and this might explain why a higher volume of NCM-eluted extract was required for Cla in comparison with CMBV.

The simplified protocol for nucleic acid extraction described in this study does not use liquid nitrogen, which is not readily available in many regional laboratories of India and probably in other developing countries. Because the spotted membranes prepared without the use of liquid nitrogen provided an efficient amplification of CMBV and Cla, even after storage for 60 days at room temperature, the membranes spotted with citrus sap can be transported over long distances from citrus orchards to a well-equipped central laboratory for detection of these pathogens without compromising the reliability of the detection.

After modification of some parameters from a previously reported methodology (Singh et al. 2006), the resulting method, evaluated in the present study for the preparation of nucleic acid templates, is reported for the first time for a DNA virus such as CMBV and the fastidious greening bacterium in citrus. Because the initial protocol has already been successfully applied to several ornamental plants with variegated and pigmented foliages containing mucilaginous and phenolic compounds (Singh et al. 2006), the modified one may be applicable to other woody plant species.

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